IMPACT OF HYPERINSULINEMIC PRIMING ON MACROPHAGE ACTIVATION

An Undergraduate Research Scholars Thesis

By

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ABSTRACT

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The impact of hyperinsulinemia on adipose tissue inflammation has been studied for decades in order to try and obtain a better understanding of the underlying effects of cardiovascular diseases and diabetes mellitus. Despite the medical interest in this subject and how adipose tissue macrophages are central to the development of adipose tissue inflammation, how these cells directly respond to the pathological dosages of insulin has yet to be proved. Inside adipose tissue macrophages (ATMs), despite the expression of the molecules mediating insulin signaling in other cell types, it is relatively unknown what direct impact insulin has on these ATMs who contribute to both systemic and adipose tissue inflammation and insulin resistance. In this study, the direct impacts of hyperinsulinemia are addressed by subjecting bone marrow derived macrophages (BMDMs) to a state of hyperinsulinemia in order to examine the direct impact that insulin has on macrophage activation through the comparison of the expression of genes related to macrophage activation by quantitative real time-polymerase chain reaction (qRT-PCR). Next the importance of prolonged exposure to pathological insulin dosages with regards to classical macrophage activation are examined by comparing the gene expression of BMDMs that were subjected to a 24 hour insulin priming period to that of BMDMs that were first exposed to insulin at the time of macrophage activation. Then, in order to understand how insulin enhances

classical macrophage response, the expressions of Toll-Like Receptor genes are examined. Finally the results from BioPlex analysis are used to determine the cell signaling protein expression and phosphorylation through which insulin achieves this impact on the macrophage cell. The results showed that prolonged exposure to pathological insulin levels results in an increase in the expression of genes associated with classical, proinflammatory macrophage activation, and this effect does not appear to be the result of an increase in Toll-Like Receptors that respond to proinflammatory stimuli. Lastly an enhanced decrease in the phosphorylation ratio of the Protein Kinase B pathway suggests that insulin resistance applies to macrophages in a manner similar to other insulin targets in the body. This project proves how critical the understanding of insulin-regulated macrophage responses is to studying hyperinsulinemia-related metabolic disorders such as diabetes.

NOMENCLATURE

ATMs	Adipose Tissue Macrophages
DIO	Diet-Induced Obesity
BMDMs	Bone Marrow-Derived Macrophages
M1	Classical Macrophage Activation
M2	Alternative Macrophage Activation
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
IRS	Insulin Receptor Substrate
ERK	Extracellular-signal-Regulated Kinases
AKT	Protein Kinase B

CHAPTER I

INTRODUCTION

Hyperinsulinemia and Obesity

Obesity occurs when caloric intake exceeds caloric expenditure, resulting in the growth of adipose tissue. In the high-fat-diet-induced obese mice, a 20-30% increase in bodyweight can be observed early on. At this point hyperglycemia and hyperinsulinemia can be observed, resulting in systemic and local insulin resistance in adipose tissue, a pre-diabetic state[1], and ultimately type II diabetes.

Macrophages in Adipose Tissue

Adipose tissue is made up of adipocytes and a number of stromal cells, including progenitor cells and infiltrated immune cells [2]. Adipose tissue macrophages (ATMs) make up a major portion of the stromal cells and are a major regulator of the adipose tissue, and therefore are critical in the development of insulin resistance in adipocytes by impairing the insulin sensitivity of adipocytes with secreted proinflammatory cytokines in the context of obesity. In the adipose niche of the high-fat-diet-induced obesity mice model, hyperinsulinemia as well as ATM-secreted proinflammatory cytokines are reported to be a contributing factor to adipose tissue inflammation[1]. In addition, there is an increase in the number of infiltrated immune cells inside the adipose tissue experiencing diet induced obesity. Exposed to the proinflammatory factors from adipocytes which are in an inflammatory status in this obese context, ATMs are reported to be in a classically activated proinflammatory state that causes chronic inflammation through the JNK and NF-κB pathways that regulate cytokine activity and lead to type II diabetes and other

cardiovascular and metabolic disorders[3-6]. In other words, hyperinsulinemia-induced inflammatory adipocytes activate ATMs classically which consequently secrete proinflammatory cytokines to reinforce adipocyte inflammation; this positive feedback loop has been proposed as the mechanism behind hyperinsulinemia-induced diseases.

Insulin and Macrophages

Despite the impact of insulin on adipocytes, the understanding of the direct impact of insulin on ATMs is not unimportant, but is still in its infancy. Research has shown that most insulin signaling molecules are present inside macrophages but their purpose is largely unknown[7]. In more general function insulin has been shown to inhibit macrophage apoptosis through a Phosphoinositide 3-kinase (PI3-kinase) dependent pathway[8], and also can stimulate the proinflammatory cytokine TNF-α production, though the pathway is not yet known[9].

CHAPTER II

METHODS

Murine Bone Marrow Macrophage Preparation

Total bone marrow cells from mouse femur and tibia bones are harvested and kept in 2% fetal bovine serum completed Iscove's Modified Dulbecco's Media. After erythrocyte lysis with ammonium chloride, cells are seeded in plates in the presence of GM-CSF (15% L929 cell supernatant is used as the source). Maturation of BMDMs is evaluated by flow cytometry after 7 day culture and a purity of minimum of 90% of $CD11b^{+}F4/80^{+}$ cells is used as naïve macrophages for later steps.

Macrophage Activation

Prior to macrophage activation, the prepared BMDMs are kept in Iscove's Modified Dulbecco's Media for 48 hours. During activation BMDMs are treated with either lipopolysaccharide (10 μg/mL) and interferon-γ (20 ng/mL) or IL-4 (20 ng/mL) and IL-13 (5 ng/mL) to obtain classical (M1) or alternative (M2) activation respectively. These BMDMs are exposed to the stimuli for five hours before harvest and collection of messenger RNA, or 24 hours before harvest and collection of proteins.

Insulin Priming

BMDMs are exposed to insulin (5.3 μg/mL) during the 24 hours prior to macrophage activation in order to accustom the cells to a state of hyperinsulinemia similar to the environment inside the

adipose tissue of obese C57BL/6J mice. These macrophages would then be activated and samples would be harvested in accordance with the previous step.

Measuring Gene Expression

Messenger RNA samples were tested via quantitative real-time polymerase chain reaction (qRT-PCR). Primers were chosen to measure the expression of genes associated with either M1 or M2 activation, and are listed in Table 1.

Table 1
Primers used for measuring gene expression

Primer	Gene function	Sequences (Sense, Antisense)
TNF-α	M1	5’ – TTGTCTACTCCCAGGTTCTCT – 3’ 5’ – GAGGTTGACTTTCTCCTGGTATG – 3’
IL-1β	M1	5’ – TGGACCTTCCAGGATGAGGACA – 3’ 5’ – GTTCATCTCGGAGCCTGTAGTG – 3’
IL-6	M1	5’ – ACAAAGCCAGAGTCCTTCAGAGAG – 3’ 5’ – TTGGATGGTCTTGGTCCTTAGCCA – 3’
ARG 1	M2	5’ – ACCTGGCCTTTGTTGATGTGCCCTA – 3’ 5’ – AGAGATGCTTCCAACTGCCAGACT – 3’
PPAR γ	M2	5’ – TCATGACCAGGGAGTTCCTC – 3’ 5’ – CAGGTTGTCTTGGATGTCCTC – 3’
IRF-4	M2	5’ – GAACGAGGAGAAGAACGTCTTC – 3’ 5’ – GTAGGAGGATCTGGGCTTGTCGA – 3’
TLR2	Receptor	5’ – CACTATCCGGAGGTTGCATATC – 3’ 5’ – GGAAGACCTTGCTGTTCTCTAC – 3’
TLR4	Receptor	5’ – AATGAGGACTGGGTGAGAAATG – 3’ 5’ – GCAATGGCTACACCAGGAATA – 3’
GAPDH	House-keeping gene	5’ – TGCACCACCAACTGCTTAGC – 3’ 5’ – GGCATGGACTGTGGTCATGAG – 3’

Insulin Priming Experiments

Bone marrow derived macrophages are separated into four different groups. Each group is exposed to stimuli as stated in Table 2. Groups are divided by whether they were exposed to

insulin during the 24 hour priming period, the 5 hour activation period, and whether or not they are classically activated. These mRNA is then harvested and examined using qRT-PCR.

Table 2
Macrophage groups for priming examination

Group	24 Hour Insulin Priming	5 Hour Inulin Exposure	M1 Activation
1	Yes	Yes	No
2	Yes	Yes	Yes
3	No	Yes	Yes
4	No	No	Yes

Examining Insulin Signaling Pathways

To investigate the insulin-triggered cell signaling pathways and their interplay with M1 or M2 stimuli, I examined the defined insulin pathways that have been proven in adipocytes first. Protein kinase B, known as Akt, is a serine/threonine-specific protein kinase that plays a critical key role in insulin signaling pathways. After total proteins are extracted with cell lysis buffer, they are examined using Bio-Plex analysis.

CHAPTER III

RESULTS

Insulin Enhance LPS-induced Classical Macrophage Activation

I examined the impact of insulin on classical and alternative macrophage activation by measuring gene expression through qRT-PCR analysis. Murine BMDMs exposed to levels of insulin that mimicked hyperinsulinemia in mice showed a significant increase in expression of genes associated with classical macrophage activation (Figure 1).

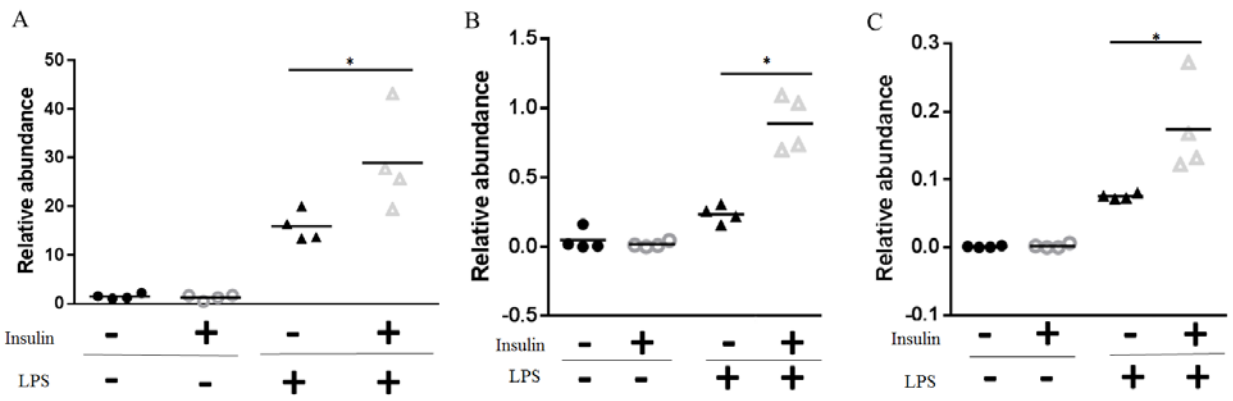

Figure 1. Insulin treatment enhances macrophage classical activation. Exposure of bone marrow derived macrophage cells to insulin prior to macrophage classical activation induced pro-inflammatory gene expression (A) *TNF-α*, (B) *IL-1β*, and (C) *IL-6* signfinicantly. Gene expression is normalized with GAPDH house-keeping gene. Data are presented as mean ± SEM; n=4. Statistical analysis was performed by one-way analysis of variance (ANOVA) with post-hoc tests *, $p<0.05$.

Insulin Priming is Critical to Augment Classical Activation

Next, in order to demonstrate the necessity of insulin priming in the process of macrophage activation, I examined the gene expression of four different groups of macrophage cells through qRT-PCR. I observed that the BMDMs which received insulin treated medium 24 hours prior to macrophage activation showed a significant increase in the expression of the genes associated with classical macrophage activation over those that only received insulin during the five hour activation period (Figure 2).

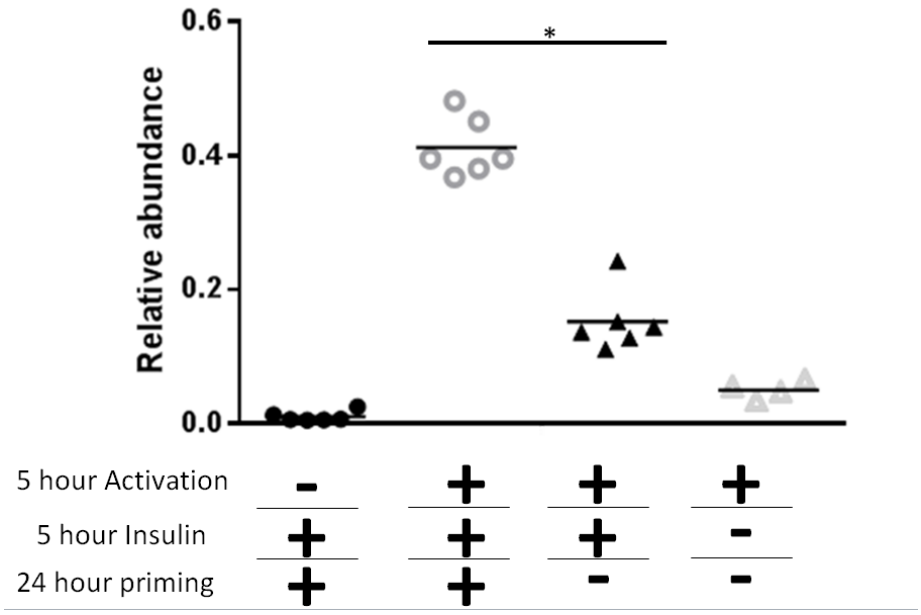

Figure 2. Insulin priming is critical to enhance macrophage classical activation. Macrophages showed a significant increase in *IL-1β* gene expression when primed with insulin during the 24 hours prior to macrophage activation. Gene expression is normalized with GAPDH house-keeping gene. Data are presented as mean ± SEM; n=4. Statistical analysis was performed by one-way analysis of variance (ANOVA) with post-hoc tests *, $p<0.05$.\

Insulin does not Enhance Classical Macrophage Activation through Increasing TLR Expression

In order to determine the cause of the enhanced classical macrophage activation, I examined the expression of the genes associated with expression of Toll-Like Receptors (TLR) through qRT-PCR. I did not observe an increase in the expression of TLR in the presence of insulin; these results support the idea that insulin does not enhance classical activation through increased TLR expression (Figure 3).

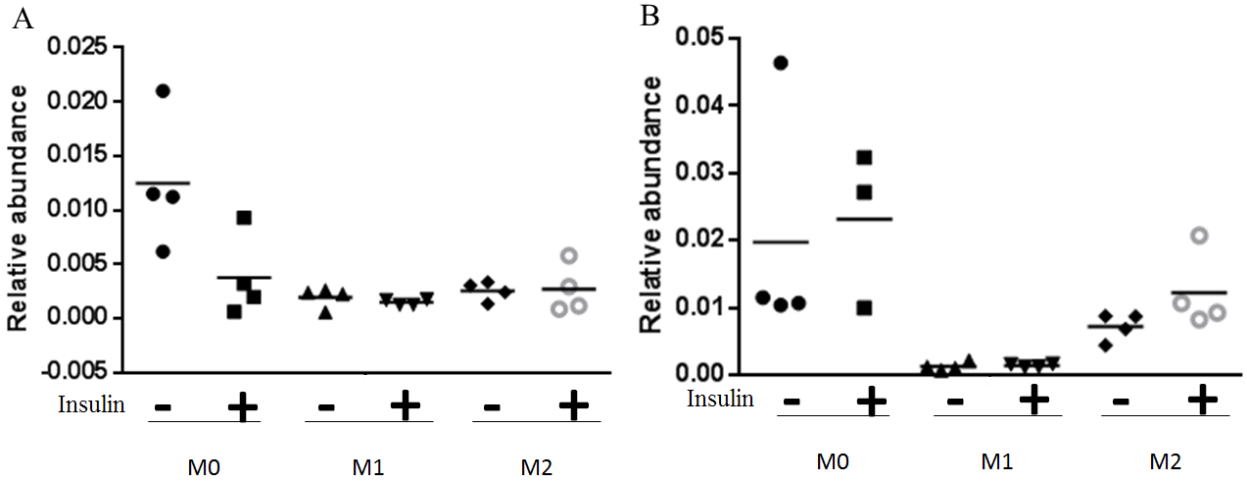

Figure 3. The effect of insulin on expression of Toll-Like Receptors. (A) Exposure to insulin decreases the expression of *TLR2* but shows no change when followed by classical or alternative activation. (B) Exposure of insulin shows no significant changes to *TLR4* expression. Gene expression is normalized with GAPDH house-keeping gene. Data are presented as mean ± SEM; n=4. Statistical analysis was performed by one-way analysis of variance (ANOVA) with post-hoc tests *, $p<0.05$.\

Insulin Priming Enhances the Classical Activation-Suppression of the Insulin Signaling Pathway

Under hyperinsulinemic conditions, it has been reported that obese subjects are undergoing local and systemic insulin resistance in insulin targeted cells including muscle and adipocytes.

Therefore I further examined if macrophages exposed to hyperinsulinemic conditions have been stressed in a way that affects insulin sensitivity. The results shown in Figure 4 show a decrease in Akt phosphorylation ratio in M1 macrophages and a more suppressed result in insulin-primed M1 macrophages by Bio-Plex analysis.

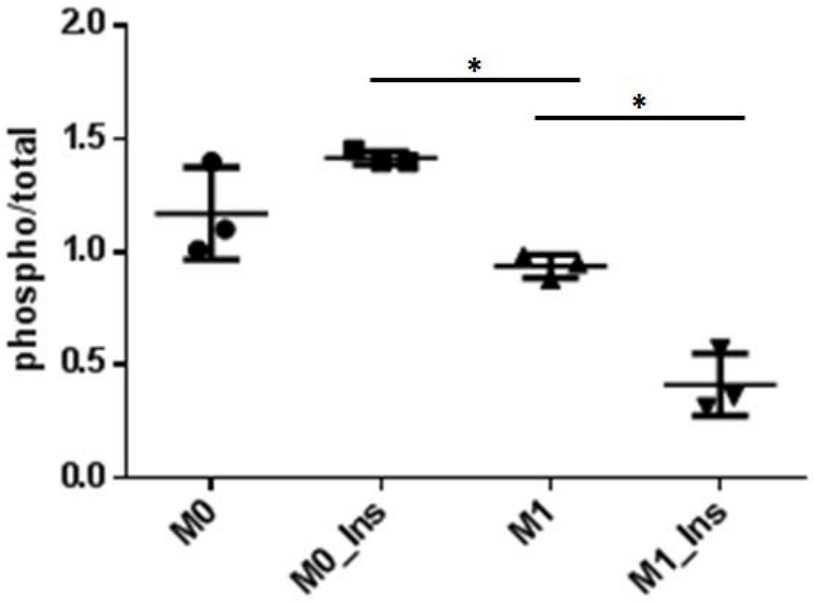

Figure 4. The effect of insulin on phosphorylation ratio of the Akt pathway. BMDMs undergoing classical activation show a decrease in phosphorylation ratio of Akt, and the group of insulin priming following by classical activation shows a stronger suppression. Data are presented as mean ± SEM; n=4. Statistical analysis was performed by one-way analysis of variance (ANOVA) with post-hoc tests *, $p<0.05$.\

CHAPTER IV

DISCUSSION

Despite the important role macrophages play in developing insulin resistance in the body, many of the direct effects of insulin on their function are still unknown. To bridge this gap in our understanding of insulin and insulin resistance, I exposed bone marrow derived macrophages to insulin and then utilized current technologies in order to examine its impact on macrophage activation. I primarily examined insulin's impact on classically activated macrophages, due to their increased presence in the obese tissues that experience a hyperinsulinemic environment, and the already existing links between classical macrophage activation and the development of insulin resistance in the body. My findings show that hyperinsulinemic conditions greatly enhance the response of bone marrow derived macrophages to pro-inflammatory stimuli.

In order to demonstrate that the continual exposure to insulin is necessary for enhanced M1 response, I measured the response of BMDMs to the same stimuli both with and without a 24 hour priming period prior to activation. The results indicate that prior exposure to insulin significantly increases the response to pro-inflammatory stimuli, and that inflammation in the body is exaggerated by the prolonged stated of hyperinsulinemia.

I then began to examine how insulin might work to achieve this enhanced M1 response, and measured the impact that insulin has on the Toll-Like Receptors in the membrane. Exposure to insulin prior to classical activation showed no significant change in the expression of genes coding for the TLRs. This is evidence to believe that the enhanced responses are not related to

the initial detection of pro-inflammatory stimuli, and that the cause of increased response to these stimuli occurs elsewhere in the cell.

I then detected the impact of insulin on the Protein Kinase B pathway in the macrophages, where I observed a decrease in the phosphorylation ratio in response to M1 stimuli. This exciting finding suggests that insulin resistance not only applies to the regular insulin targets that regulate glucose uptake but also to macrophages controlling inflammatory response.

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